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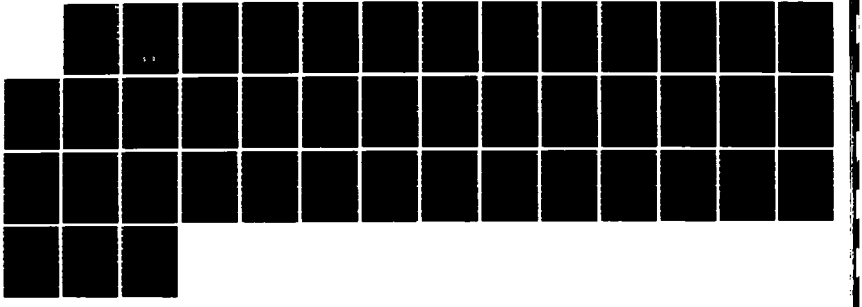

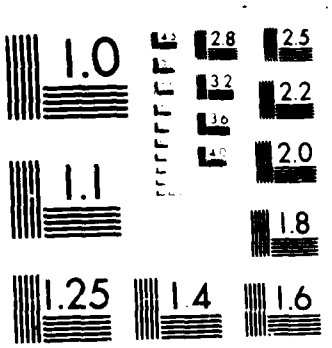

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ENZYME MINI-TEST FOR FIELD IDENTIFICATION OF *LEISHMANIA* ISOLATES FROM U.S. MILITARY PERSONNEL

Annual Report

RICHARD D. KREUTZER, Ph.D.

15 August 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

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Youngstown State University
Youngstown, Ohio 44555

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It is possible to identify Leishmania isolates by cellulose acetate electrophoresis (CAE) of up to 29 enzyme activities. Certain of these enzymes are polymorphic within a subspecies and therefore of limited value for identification; others are monomorphic and have taxonomic significance. Once large numbers of isolates from various geographical areas have been characterized and monomorphic enzymes identified, a simple, rapid, accurate field type identification test can be devised.

Data for up to 29 enzymes have been obtained from about 400 Leishmania isolates by CAE. Among the isolates were two groups designated as reference strains. Enzyme profiles have been established for many Leishmania subspecies based on CAE data from over 20 widely distributed isolates and reference strains. The subspecies profiles thus established are as follows: L. branziliensis panamensis (LBP - 104 isolates), L. b. braziliensis (LBB - 56), L. b. guyanensis (LBG - 28), L. mexicana mexicana/pifanoi (LMM/P - 43/2), L. m. amazonensis/garnhami (LMA/G - 47/2), L. m. aristedesi (LAR - 1), L. m. enrietti (LME - 2),

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L. donovani (LD - 28), L. chagasi (LC - 27), L. major (LMJ - 32), L. tropica (LT - 25), L. aethiopica (LAE - 7), L. hertigi hertigi (LHH - 4), L. h. deani (LHD - 1), L. gerbilli (LGE - 1). The subspecies previously referred to as "L. m. venezuelensis" was actually LMM/P, and the LMM/LMA group of isolates was LMA/G. No consistent CAE differences have been noted between LMM and LMP or between LMA and LMG; therefore, they have been noted as LMM/P and LMA/G respectively. Data from more isolates of LMP and LMG are needed.

As the number of isolates studied from a particular geographical area increased so did the diversity of Leishmania subspecies; therefore, to obtain an accurate estimate of the Leishmania fauna in an area many isolates must be identified. Routine CAE identification was performed for certain WRAIR contractees and other sources. When appropriate, data from these isolates were included in subspecies enzymes profiles. Isolates obtained by the P.I. in Colombia and identified by CAE have been sent to and have been added to the WRAIR cryobank. It was suggested that preliminary and confirmatory CAE identification be made a part of all WRAIR Leishmania studies. A second and more complete draft of the mini-test for Leishmania identification was included. The enzyme profile data from 400 isolates suggest that accurate CAE identification can be made from data of GPI, MPI and 6PGDH. The buffer and the stain components for these enzymes can be prepackaged, and data can be obtained from 10^6 Leishmania cells. Combined biochemical data were analyzed and the results were presented in a dendrogram based on CAE similarities and differences within and between Leishmania subspecies complexes. The CAE data which have been generated in this and other studies have been compiled in a computer program so they are readily available to personnel at WRAIR.

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Problem

Leishmania isolates from any source can be accurately and rapidly identified by cellulose acetate electrophoresis (CAE). Studies using 29 enzyme activities have shown that certain enzymes are polymorphic and of limited value in identification, while others are monomorphic and have taxonomic significance. It is, therefore, necessary to characterize isolates from a series of geographic areas by examining data from a number of enzymes to find a few monomorphic enzymes that can be used in a simple, rapid, accurate field identification test.

Background

Electrophoretic study of gene-enzyme systems represents a powerful tool in systematics, genetics, ecology, ethology and applied biology. Studies on the systematic value of electrophoretic data reveal high levels of genetic similarity between conspecific populations, with up to 85% identity of their loci, while the percent of genetic similarities among closely related species are usually much lower.[1-4]

Recently enzyme electrophoresis has been used to identify parasites of the genus *Leishmania*[5-11] One study reported that 21% of the enzymes tested from particular subspecies were polymorphic.[11] This suggests that isolates which have profiles greater than 75% identical are the same subspecies, and isolates which are less than 75%

identical are parasites from different subspecies. The enzyme profiles of some isolates in this study were identical, but in most cases, the profiles among isolates in a given subspecies were different. Leishmania could be grouped into at least five major complexes according to enzyme profiles, braziliensis, mexicana, donovani, tropica and hertigi. These designations for the most part are consistent with the taxonomic categories used by others.[12] Within these complexes the isolates could be grouped into subspecies with varying levels of allozyme identity. It has been reported that the L. braziliensis complex is composed of two possibly three (L. b. guyanensis) subspecies, L. b. panamensis and L. b. braziliensis. The L. mexicana complex has at least three subspecies, L. m. mexicana, L. m. amazonensis and L. m. aristidesi. Minimal numbers of L. tropica and L. donovani had been examined and subspecies remained to be delineated. These data were consistent with those obtained by the principal investigator in an earlier less comprehensive study. Results of this study demonstrated that electrophoresis can be used for rapid and accurate subspecific identification of Leishmania.[11]

The data which have been reported on enzyme profiles of leishmanial groups have been obtained from small numbers of isolates. Among known species there is a certain amount of naturally occurring enzyme polymorphism. Isolates which

have been identified as belonging to the same group by classical methods and their identification confirmed by CAE do have slight genetic differences as noted from their individual enzyme profiles. This type of population polymorphism is to be expected and has been noted in all groups of organisms which have been studied by electrophoresis.[1-4]

A taxonomic enzyme profile must include as much information on polymorphism as can be obtained. The problems which can result from establishing taxonomic enzyme profiles from small numbers of isolates are noted in the following example. If L. donovani WR130 from Khartoum and L. donovani WR352 from India are compared, 68% of 25 enzymes would be identical by CAE, but when the WR352 profile is compared to ten other L. donovani isolates, the levels of identity are greater. Furthermore, if the enzyme analysis were confined to MDH, ICD, GOT, ALAT, AK1, EST, ACP1, MPI (Table 1) there would have been 0% identity and the two isolates would have been considered as two different species complexes. Therefore, taxonomic profiles must be based on many systems and on data from a large number of isolates representing the entire geographical distribution of the group.

The identification of Leishmania based on 29 genetic loci produces more accurate results; however, it should be possible to make a rapid and accurate identification using

only two or three enzymes once the geographic area of isolation is known. For example the WRAIR isolates WR209 (L. b. panamensis), WR359 (L. b. braziliensis), WR225 (L. m. amazonensis), WR381 (L. m. mexicana), and WR285 (L. chagasi) are all from Panama. These isolates can be separated as follows:

GPI	209 and 359 are identical. This pair differs from 225, 381 and 285 which differ from one another.
ALAT	359 and 285 are identical. This pair differs from all others.
MPI	Each differs one from another.

Isolates from the Ft. Sherman area in Panama would require the use of WR209, WR225 and WR285 as reference subspecies. Any of the three enzymes could be used to establish that an unknown is L. m. amazonensis and not the other subspecies, while MPI could be used to establish that the unknown as either L. b. panamensis or L. b. braziliensis. From this example it is evident that baseline studies on isozyme polymorphism are required to select the few taxonomically relevant enzymes to be used in a simplified identification procedure.

Approach

This study was designed to establish methods which the clinical laboratory technician can follow in the field to rapidly and accurately identify leishmanial isolates from

U.S. military personnel. At present, emphasis is placed on identification of New World and Kenyan isolates in support of military operations in Central America and USAMRU-Kenya.

Isolates which are grown in any medium that can support sufficient growth of promastigotes were examined by CAE using up to 29 enzyme systems. At least 20 different isolates of each subspecies with adequate histories are studied to obtain information on natural polymorphism. Standard enzyme profiles have been established in a previous study,[11] and unknown isolates were compared to these standard profiles. The enzyme profiles of each subspecies were compiled to determine which enzymes have taxonomic value.

Although the procedure for CAE identification of leishmanial isolates is simple, it should be possible to further reduce the time and effort required to rapidly and accurately identify isolates. A simple kit designed for a particular geographic area could be assembled. This kit will contain instructions, buffers, stains, controls, and information on interpretation of results.

Summary of Previous Year Progress

Approximately 200 Leishmania isolates have been characterized for up to 29 enzymes by CAE. About 150 of these isolates were primarily isolates from the WRAIR cryobank and the remainder were from individual WRAIR

investigators or contractees. Among the primary isolates were those designated by WHO as reference strains. Enzyme profiles based on reference strain isolates have been established for L. braziliensis panamensis (LBP), L. b. braziliensis (LBB), L. b. guyanensis (LBG), L. mexicana mexicana (LMM), L. m. amazonensis (LMA), L. m. aristedesi (LAR), L. pifanoi (LMP), L. m. enriettii (LME), L. garnhami (LMG), L. donovani (LD), L. chagasi (LC), L. major (LMJ), L. tropica (LT), L. aethiopica (LAE), L. hertigi hertigi. It has been noted that the numbers of Leishmania subspecies identified from a given area is related to the numbers of isolates examined; i.e. as more isolates from a particular geographic locale were studied the number of subspecies identified increased. A group of isolates from human hosts from Central America, some with simple cutaneous and others with diffuse cutaneous leishmaniasis (DCL), were over 75% identical, and therefore samples from one Leishmania subspecies. These data suggest that DCL could be a result of host response to the parasite. It is possible that these isolates are LMM. Identification of isolates should precede studies on Leishmania; furthermore, confirmatory CAE identification should be made as such studies progress. Such identifications insure the reliability of the data obtained. Preliminary data indicate that two strains of visceral isolates, one derived from the other and one susceptable and the other resistant to SB^{+5} treatment can

re separated by Ab. It is possible that specific enzyme polymorphisms might be related to drug susceptibility. The test leading to accurate and rapid identification of Leishmania isolates requires analysis of enzymes which produce distinctly migrating bands for each subspecies, are monomorphic and are simple to run. The enzymes which appear to meet these requirements are GOT, GPI, GSR_1, GSR_2, ICD, MDH, MPI and 6PGDH. Simplified CAE conditions for these enzymes have been established. A preliminary draft for the test has been proposed which includes the enzymes GPI, MPI, 6PGDH and GSR_2. The data generated in this study were combined in a dendrogram based on CAE similarities and differences within and between Leishmania species complexes. A new enzyme GSR_2, and conditions for its CAE were reported. Technicians in the Leishmania section at WRAIR are already using the information provided in this report.

Progress

Isolates identified

In this third year of the project an additional 165 Leishmania isolates from both New and Old world have been identified (Table 2). Recently (June, 1985) CAE study has been initiated on a new series of World Health Organization (WHO) reference isolates WR657-685. Data from eight enzymes are available, and the preliminary enzyme profiles of these reference isolates are identical to the established profiles

of each subspecies. There was one important development among the data from these isolates. Previous reports have noted an unknown mexicana complex subspecies "L. m. venezuelensis", ("LMV"), and that it was not possible to separate L. m. amazonensis (LMA) and L. m. mexicana (LMM). Among these reference strains were two LMA isolates (WR669-670) and two LMM isolates (WR667-668). The profiles of the two LMA reference isolates were identical (for eight enzymes) to the group previously identified as LMA/LMM, and the profiles of the two LMM isolates were identical (for eight enzymes) to the group previously identified as "LMV". The established CAE profile for "LMV" is the LMM profile, and the LMA/LMM profile is the LMA profile. In addition preliminary CAE enzyme profiles have been established for L. hertigi deani (LHD) and L. gerbilli (LGE). The profile of LHD has some similarity to but is distinct from that of L. h. hertigi (LHH), and the LGE profile has little similarity to the established profile of any other subspecies.

In this study enzyme data from over 20 isolates of New World visceral, L. chagasi (LC), and Old World visceral, L. donovani (LD), have been obtained. For most of the enzymes studied (Table 1) both groups are identical and monomorphic; however, for ASAT, GOT and MDH the allomorph frequency differences between the two groups of isolates are as follows:

	ASAT*		GOT*		MDH	
	Fast	Slow	Fast	Slow	Fast	Slow
LD	0.69	0.31	0.50	0.50	0.31	0.69
LC	0.00	1.00	0.00	1.00	0.00	1.00

*ASAT and GOT might be the same enzyme.

These data suggest that if a visceral isolate has the "slow" allomorph for ASAT and MDH it is only 20% likely that it is LD. Similar data are not yet available for separating L. infantum from (LI) and (LC).

At present only two isolates of L. m. garnhami (LMG) and two of L. m. pifanoi (LMP) have been studied. No consistent differences have been noted between the profiles of LMA and LMG or the profiles of LMM and LMP; therefore, until larger samples of LMG and LMP are studied it is not possible to separate these pairs.

The profile of L. b. guyanensis (LBG) is very similar to that of L. b. panamensis (LBP), but both profiles are quite different from the profile of L. b. braziliensis. The profiles of LBP and LBG differ as follows:

ACP				MDH				ME				6PGDH			
LBP		LBG		LBP		LBG		LBI		LBG		LBP		LBG	
A	1.00*	A	0.00	A	0.68	A	0.00	A	0.05	A	1.00	A	0.00	A	1.00
B	0.00	B	1.00	B	0.32	B	1.00	A	0.84	B	0.00	B	0.33	B	0.00
C	1.00	C	0.00					C	0.06	C	0.00	C	0.67	C	0.00
D	0.00	D	1.00												

*Allomorph frequencies.

Therefore, data from ACP and 6PGDH can be used to identify isolates of these two subspecies.

In summary CAE enzyme profiles have been established for LBP, LBB, LBG, LMM/LMP, LMA/LMG, L. m. aristedesi (LAR), L. m. enriettii (LME), LD, LC, L. major (LMJ), L. tropica (LT), L. aethiopica (LAE), LHH and L. h. deani (LHD) and LGE.

Leishmania diversity

As noted in previous reports there appears to be a direct relationship between the number of isolates characterized from a particular geographical locale and the number of subspecies identified in that area. Among 83 isolates from Panama, 37 from Brazil, 50 from Colombia, and 14 from Belize there is a higher level of subspecies diversity than among isolates from other geographical regions which have been less vigorously studied (Table 3). These supporting data on the relationship between diversity and numbers of isolates examined emphasize the need to examine large numbers of isolates from all geographical areas from which leishmaniasis has been reported to obtain an accurate picture of the degree of Leishmania diversity.

Routine identification for WRAIR contractees

In addition to the WRAIR primary isolates, samples for CAE identification were received from and identified for individual WRAIR personnel and contractees. These are noted in Table 2 as from P. Jackson and Keithly. Other isolates

from NIH and Colombia are also identified. Certain identified Colombian isolates are now in the WRAIR cryobank. In each case the isolate identification was based on comparative data from previously identified WHO reference strains. In certain cases the CAE identification was made prior to initiation of other studies on the isolate and later follow up confirming identification was made during the course of studies by the contractee. It is suggested that groups involved in Leishmania research either establish CAE identification in their own laboratories or that identification confirmation be made at this laboratory prior to study. In addition CAE identification should be made a standard procedure in each project. Preliminary and confirmatory CAE identification is considered necessary, because certain isolates received at this laboratory from various sources and labeled as a particular species were identified by CAE as being other than the indicated species or as mixed cultures. Identification by CAE is too simple and accurate not to take advantage of the process.

Mini Test

General

Isolates of a particular subspecies have a high level of enzyme similarity (over 75%), but usually no two isolates are 100% identical. The allozyme differences among isolates are the result of natural polymorphism and can be of importance when studying biological parameters other than

identification. Enzyme polymorphism can affect electrophoretic identification of Leishmania isolates. If, for example, 6PGDH were the only enzyme used for an identification, LBP, LBB and LBG could be separated even though two differently migrated bands have been observed among isolates of LBP and LBB (non-overlapping polymorphism). LMM and LMA which are also polymorphic for 6PGDH could not be separated because their polymorphism is overlapping. Then 6PGDH can identify L. braziliensis subspecies but not L. mexicana species. Enzymes chosen for a mini test identification should have either no or non-overlapping polymorphism, but ones which can separate Leishmania species and subspecies. Another consideration is the choice of enzymes which are very active (i.e. produce bands with small numbers of cells) and which are relatively simple to prepare. One monomorphic enzyme, simply prepared, with distinctly migrating bands for each Leishmania subspecies would be sufficient, unfortunately no single enzyme yet studied meets all of these requirements.

Population enzyme polymorphism is probably the most difficult parameter to determine. It requires study of many isolates (ideally 20) from the entire distributional range of the subspecies, but for many Leishmania subspecies only a few isolates from a restricted geographical area have been studied. In this study attempts were made to reduce possible errors in biochemical identification by collecting

data from many isolates with well documented (if possible already identified) histories from multiple geographical areas, Tables 2 and 4. The data from the isolates already run indicate that enzyme polymorphism is either minor or not present for the enzymes GOT, GPI, GSR_1, ICD, MDH, MPI and 6PGDH; furthermore, these enzymes can be used to separate most New and Old World Leishmania. These enzymes produce good activity from small numbers of cells, and buffer/strain components have been changed so each system can be preweighed, sent through the mail and requires only the addition of distilled water prior to use (Table 5). Although there are eight enzymes noted in the table, the recommended simplified test for Leishmania identification will include only GPI, MPI, and 6PGDH.

Second draft of mini test

1. Cells needed for identification.
    A. A minimum number of 10^6 cells are needed to obtain data from GPI, MPI, and 6PGDH; however, visible masses of cells grown on and picked off blood agar medium in petri dishes without a liquid overlay are sufficient to produce activity with these enzymes.
2. Cell preparation for CAE.
    A. Separate cells from growth medium at 1,000 g for 10 min. and pour off growth medium.

B. Add 1 mL of less normal saline to the pellet (visible mass of cells).

C. Transfer saline and cells to a 2 mL nunc tube.

D. Separate cells and saline at 1,000 g for 10 min. and remove all saline using a pipette if necessary.

E. Add an appropriate amount of buffer (14 parts distilled water: 1 part cell buffer 1 or 2 - Table 5) to the cells, about 1/3 the size of the pellet or 6 uL/10^6 cells.

F. Resuspend the cells in the buffer with a vortex.

G. Rapidly freeze and thaw three times. Store 10^6 cells at -70°C until needed.

H. For large cell masses separate lysate from cell debris at 1,000 g for 10 min.

I. With a pipette remove the lysate from the cell debris and store in a nunc tube at -70°C until needed.

3. Buffer/stain preparation and conditions for CAE. See Table 5. If a pH meter is available, it is recommended that cell buffer 1 be used for GPI.

A. Buffer preparatiion.

1. Cell buffers 2-5 and reaction buffers B-G can be prepackaged, sealed and stored at room temperature indefinitely.

2. Add the buffer components to the appropriate

amounts of distilled water and stir until solved.

3. Buffers can be prepared for any final volume, the amounts noted in Table 5 are for preparation of one liter of each buffer.

B. Stain preparation.

1. Stain components can be prepackaged, sealed and stored at -30°C indefinitely.
2. 25 mL of the appropriate reaction buffer are combined with 0.5 g nobel agar and brought to boiling on a stirring hot-plate.
3. Cool to 50° C.
4. While the agar is cooling, add 25 mL of the reaction buffer to the substrate/stain components and stir until dissolved. It might be necessary to use a glass rod to break up large particles which do not readily dissolve.
5. Pour the cool (50°C) agar into the substrate stain solution and continue stirring for 5 seconds.
6. Pour the mixture into 100 x 15 mm petri dishes (5-6 dishes) and store at 4°C.
7. It is recommended that the stain plates be used as soon as possible.

4. Electrophoresis

A. Components (Helena Labs, Beaumont, Texas).

1. Tital Power Supply (Cat. No. 1500, 110V).
2. Zip Zone Chamber (Cat. No. 1283).
3. Disposable Wicks (Cat. No. 5081).
4. Super Z Applicator Kit (Cat. No. 4088).
   a. Applicator
   b. Sample Well Plate
   c. Aligning Base
5. Zip Zone Plates (Cat. No. 3023)
6. Microdispenser (1-10 uL dispenser)

B. Preparing the Zip Zone chamber.

1. Add 100 mL of cell buffer to each outer well of the zip zone chamber and apply a paper wick to each center rib.
2. If cooling is necessary, add ice cubes to the center wells. Ice should not touch the wicks.

C. Zip zone plates should be carefully placed in the membrane buffer (prevent trapping air bubbles in the plate) and soaked for at least 10 minutes prior to use.

D. Place 3 uL portions in the slots of the sample well plate and dilute as noted in Table 5.

E. Remove zip zone plates from the buffer, blott dry and place on the aligning base parallel to the second line from the top of the base.

F. Use the applicator to transfer the lysate from three sample well plates to the zip zone plate.

G. Transfer the zip zone plate to the zip zone chamber. The plate is applied lysate side down and application side to the negative side of the chamber. The plate should be perpendicular to the paper wicks. Place microscope slides on the plate to insure wick/plate contact.

H. Set appropriate time and voltage.

1. After electrophoresis, remove the zip zone plate, blot ends dry, cut the plate to fit the petri dish and apply it enzyme side down to the substrate/stain/agar plate. Carefully press out any air bubbles.

J. Monitor the plates and mark the bands as they appear. Mark the plates on the plastic side with a cryomarker.

K. After all samples have been marked, remove the plate, place it in 5% acetic acid for 30 seconds, wash in tap water and allow to air dry.

5. Controls. To identify an isolate a comparison of the unknown to a known must be made. Control isolates are any which have already been identified by electrophoresis and are prepared as noted above. It is recommended that only isolates designated as reference isolates by WHO be used as controls. The

object of the contrl is to produce a band of migration so unknown isolate bands can be compared with it. Two bands which migrate equally indicate identity. If a known isolate ard an unknown produce identically migrating bands for GPI, MPI and 6PGDH, the unknown and the known are the same Leishmania subspecies.

A. Preliminary run controls in Sample Well Plate.

1. LBB well 3.
2. LBP well 4.
3. LMA well 5.
4. LC well 6.
5. Wells 1, 2, 7, 8 for unknowns or if necessary other controls.

B. Confirming run controls. After a preliminary identification has been made, a second run should be made. Each unknown should be placed next to the suspected control. This will confirm both bands migrate identically.

6. Sequence of enzymes leading to identification (Figures of MPI and 6PGDH are not yet available).

A. Run GPI.

1. Separate (Figure 1) - LB complex subspecies - LBP, LBB, LBG; LD complex subspecies - LD, LC, LI; LMM-P; LMA-G; LAR; LT; LHH-LHD; LAE-LGE.

2. Identical band: LBP-LBB-LBG-LMJ-LMG; LD-LC-LI; LHH-LHD; LAE-LGE.

B. After GPI, run MPI.

1. Separate (in addition to data in 6A1 above) - LBB, LAE, LGE, LMJ, LME, LHH, LHD.

2. Identical band: LD complex, LBP-LBB.

C. After GPI and MPI, run 6PGDH.

1. Separate (in addition to data in 6A1 and 6B1 above) - LBP-LBB-LBG.

2. Identical band: LD complex.

D. LD complex subspecies identification.

1. Geographical area of isolation.

2. Run GOT (ASAT) and MDH - If both allomorphs are "slow", it is 35% likely that the isolate is LD or 65% likely it is LC.

3. At present no reliable CAE procedure is available to separate LI from LD or LC.

This is the second draft of the proposed simplified test. The test is a major objective of the project, and will be complete in all respects for the final report. The data from almost 500 Leishmania isolates indicate that accurate identification of most isolates can be made by study of only three enzymes, but a complete analysis of at least 25 enzymes should be made of each isolate. It is possible that enzyme polymorphisms not observed in the preliminary, three enzyme identification could correlate

with other biological parameters.

In summary it appears that the subspecies of Leishmania for which enzyme profiles are available can be separated by study of three enzymes, GPI, MPI and 6PGDH.[22] These are active enzymes for which CAE procedures have been simplified and among which only minor enzyme polymorphism has been observed. Most of these preliminary data on the simplified test have already been made available to and are being used by personnel in the Leishmania section at WRAIR for isolate identification.

Biochemical similarities among Leishmania

Data from the isolates studied have been combined and correlated. The levels of similarity and difference among subspecies has been calculated in a manner similar to that used for genetic identity and distance analysis in diploids,[1-3] (Table 6). These data can be used to produce a dendrogram or grouping of Leishmania subspecies. This type of analysis is more meaningful if it includes data from about 20 enzymes for about 20 isolates of each subspecies. The dendrogram (Fig. 2) includes some subspecies similarities for LAR, LMP, LMG, LI, LHH, LHD and LGE based on data from one or two isolates; therefore, the similarities of these subspecies must at present be considered preliminary. No CAE data are available on certain other Leishmania New or Old World subspecies such as L. b. peruviana, L. m. venezuelensis and others.

Computer analysis

The CAE data which have been generated by this and other studies have been compiled in a computer program so they are readily available to personnel at WRAIR. The program for the CAE data is menu operated and IBM compatible. Data from each Leishmania subspecies are combined into a file, and each file has columns for ID number, parasite species, date, enzyme and polymorphism. This system allows simultaneous determination of numbers of enzymes used for isolate identification, allomorphs of the isolate and a comparison of the isolate's enzyme polymorphism with the polymorphisms of all other isolates of the same subspecies. In addition it facilitates the polymorphism analysis of each subspecies, as well as providing a compiled visible picture of which enzymes remain to be tested for an isolate.

Publications and reports at meetings

Kreutzer, R. D., N. Souraty and P. B. McGreevy. 1983. New World diffuse cutaneous leishmaniasis:possibly one enzyme type. Annual meeting of Am. Soc. Trop. Med. Hyg.

McGreevy, P. B., R. D. Kreutzer, E. D. Franke, H. A. Stimson, C. N. Oster and L. D. Hendricks. 1983. Taxonomy, clinical pathology and prognosis of leishmaniasis in U. S. soldiers infected in Panama. Annual meeting Am. Soc. Trop. Med. Hyg.

Kreutzer, R. D. and N. Souraty. 1984. Accurate identification of Leishmania isolates by study of three enzymes. Annual meeting Am. Soc. Trop. Med. Hyg.

Kreutzer, R. D., N. Souraty, P. B. McGreevy and E. D. Franke. In review. A New World Leishmania which can cause either cutaneous or diffuse cutaneous leishmaniasis in human hosts. Am. J. Trop. Med. Hyg.

Chulay, J. D., C. N. Oster, P. B. McGreevy, R. D. Kreutzer and L. D. Hendricks. In review. American cutaneous leishmaniasis:clinical presentation and problems of patient management. Annals Intern. Med.

TABLE 1. Enzymes tested in this study.

Enzyme	Enzyme Abbreviation
Oxidoreductases	
Lactate dehydrogenases (1.1.1.27)	LDH
Malate dehydrogenase (1.1.1.37)	MDH
Malic enzyme (1.1.1.40)	ME
Isocitrate dehydrogenase (1.1.1.42)	ICD
Phosphogluconate dehydrogenase (1.1.1.44)	6PGDH
Glucose-6-phosphate dehydrogenase (1.2.1.49)	G6PDH
Glyceraldehyde-phosphate dehydrogenase (1.2.1.12)	$GAPDH_1$, $GAPDH_2$
Gluthione reductase (1.6.4.2)	GSR_1, GSR_2
Transferases	
Glutamate-oxaloacetate transaminase (2.6.1.1)	GOT & ASAT
Glutamate-pyruvate transaminase (2.6.1.2)	ALAT
Hexokinase (2.7.1.1)	HK
6-Phosphofructokinase (2.7.1.11)	FK
Adenylate kinase (2.7.4.3)	AK
Guanylate kinase (2.7.4.8)	GUK
Phosphoglucomutase (2.7.5.1)	PGM_1, PGM_2
Hydrolases	
Esterases (3.1.1.1)	EST
Acid phosphatase (3.1.3.2)	ACP
Peptidases (3.4.11.-13)	PEP
Peptidase D (3.4.13.9)	PEPD
Lyases	
Aldolase (4.1.2.13)	ALD
Fumerate hydratase (4.2.1.2)	FUM
Isomerases	
Mannose phosphate isomerase (5.3.1.8)	MPI
Glucose phosphate isomerase (5.3.1.9)	GPI

TABLE 2. CAE isolate identification.

WRAIR No.	Category	Parasite Species	Host Species	Source	Locality	Other Designation
2718	OWV	LD[6]	Man	USAMRU-K	Kenya	LV650
307	OWV	LD	Man	USAMRU-K	Kenya	209762/400-78
310	OWV	LD	Man	USAMRU-K	Kenya	298807
312	OWV	LD + LMM-P	Man	USAMRU-K	Kenya	307024
326	OWV	LD	Man	USAMRU-K	Kenya	321827
417	NWC	LMA-G	Hamster	LAN-SHAW	Brazil	LV79, MI841, LRCL309
PJ001[2]	NWV	LC	Man	P.Jackson/ Reed	Brazil	BA3
PJ003	NWV	LC	Man	P.Jackson/ Reed	Brazil	BA7
PJ004	NWV	LC	Man	P.Jackson/ Reed	Brazil	BA9
PJ005	NWV	LC	Man	P.Jackson/ Reed	Brazil	BA10
PJ006	NWV	LMJ	-	P.Jackson/ Reed	Brazil	BA11
PJ007	NWV	LC	Man	P.Jackson/ Reed	Brazil	BA12
PJ008	NWV	LC	Man	P.Jackson/ Reed	Brazil	BA13
CL002B	NWC	LMM-P	Man	Corredor	Colombia	MHOM/CO/84/CL002B
CL005B	NWC	LMM-P	Man	Corredor	Colombia	MHOM/CO/84/CL005B
CL013B	NWC	LMM-P	Man	Corredor	Colombia	MHOM/CO/84/CL013B
CL018A	NWC	LMM-P	Man	Corredor	Colombia	MHOM/CO/84/CL018A
CL020A	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/84/CL020A
CL021	NWC	LPIF?	Man	Corredor	Colombia	MHOM/CO/84/CL021
CL022	NWC	LBB	Man	Corredor	Colombia	MHOM/CO/84/CL022
CL023	NWC	LBB	Man	Corredor	Colombia	MHOM/CO/84/CL023
CL024	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/84/CL024
CL026	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/84/CL026
CL027	NWC	LPB	Man	Corredor	Colombia	MHOM/CO/84/CL027
N032[4]	NWC	LBB	Man	Sacks	-	Torrez
N033	NWC	LB and LM	Man	Sacks	-	Broos
N034	NWC	LBB	-	Sacks	-	WR608
N035	NWC	LBB	-	Sacks	-	WR605
N036	NWC	LBB	-	Sacks	-	WR604
N037	NWC	LBB	-	Sacks	-	WR603
N039	OWV	LD	Man	Neva	India	Mongi
N040	OWC	LAET	Man	Neva	Ethiopia	Hulum
N041	OWC	LAET	Man	Neva	Ethiopia	Kassahun
N042	OWC	LAET	Man	Neva	Ethiopia	Gede
N043	OWC	LAET	Man	Neva	Ethiopia	Degu
N044	OWC	LAET	Man	Neva	Ethiopia	Kassaye
N045	OWV	LD	Man	Neva	India	Sebalek
N046	OWV	LD	Man	Neva	India	Child
N047	NWC	LMM-P	Man	Neva	-	Morton

TABLE 2. CAE isolate identification - continued.

WRAIR No.	Category	Parasite Species	Host Species	Source	Locality	Other Designation
047	OWC	LT	Man	Bray	Bengal	L117RG, LRCL160, WR221B
221	OWC	LT	Man	Bray	Bengal	L117RG, LRCL160, WR047
339	OWV	LD	Man	USAMRU-K	Kenya	1336
350	OWC	LT	Man	-	Iraq	LRCL32, LV142, WR296
353	OWC	LMJ	Tatera sp.	Baringo	Kenya	LRCL119, LV181, LUMP1972
372	OWV	LD	Man	USAMRU-K	Kenya	L89
375	OWV	LD	Man	USAMRU-K	Kenya	IDH1908, 380544
576	OWC	LT	-	-	-	-
625	NWC	LBP	Man	WRAIR	Panama	-
626	NWC	LBP	Man	WRAIR	Panama	-
626B	NWC	LBP	Man	WRAIR	Panama	-
627	NWC	LBP	Man	WRAIR	Panama	-
637	NWC	LBP	Man	WRAIR	Panama	-
638	NWC	LBP	Man	WRAIR	Panama	-
639	NWC	LBP	Man	WRAIR	Panama	-
640	NWC	LBP	Man	WRAIR	Panama	-
641	NWC	LBP	Man	WRAIR	Panama	-
642	NWC	LBP	Man	WRAIR	Panama	-
642B	NWC	LBP	Man	WRAIR	Panama	-
643	NWC	LBP	Man	WRAIR	Panama	-
644	NWC	LBP	Man	WRAIR	Panama	-
645	NWC	LBP	Man	WRAIR	Panama	-
646	NWC	LBP	Man	WRAIR	Panama	-
654	NWC	LBP	Man	WRAIR	Panama	-
CL001B	NWC	LBP	Sandfly	Corredor	Colombia	ITRA/CO/81/CL001B
CL002C	NWC	LMM-P	Sandfly	Corredor	Colombia	MHOM/CO/84/CL002C
CL005C	NWC	LBP	Sandfly	Corredor	Colombia	MHOM/CO/84/CL005C
CL006B	NWC	LBP	Sandfly	Corredor	Colombia	ITRA/CO/81/CL006B
CL015B	NWC	LBP	Sandfly	Corredor	Colombia	ITRA/CO/84/CL015B
CL018B	NWC	LMM-P	Man	Corredor	Colombia	MHOM/CO/84/CL018B
CL031	NWC	LBB	Man	Corredor	Colombia	MHOM/CO/84/CL031
CL033	NWC	LBB	Man	Corredor	Colombia	MHOM/CO/84/CL033
CL035	NWC	LBB	Man	Corredor	Colombia	MHOM/CO/84/CL035
CL038	NWC	LBB	Man	Corredor	Colombia	MHOM/CO/84/CL038
CL039	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/84/CL039
CL041	NWC	LBB	Man	Corredor	Colombia	MHOM/CO/84/CL041
CL042	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/84/CL042
CL043	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/84/CL043
CL044	NWV	LC	Man	Corredor	Colombia	MHOM/CO/84/CL044
KO30[5]	NWC	LBG	-	Keithly	-	-
KO31	NWC	LBG	-	Keithly	-	-
KO32	NWC	LBG	-	Keithly	-	-
KO33	NWC	LBG	-	Keithly	-	-
KO34	NWC	LBG	-	Keithly	-	-
KO35	NWC	LMA-G	-	Keithly	-	-
KO36	NWC	LMA-G	-	Keithly	-	-

TABLE 2. CAE isolate identification - continued

WRAIR No.	Category	Parasite Species	Host Species	Source	Locality	Other Designation
126	NWC	LMM-P	Man	WRAIR	Texas	WR127, ATCC30883, LV468
363	NWC	LMA-G	Man	WRAIR	Brazil	LBO16C, M1287
367N	NWC	LBB	Man	WRAIR	Brazil	LTB0014, WR386
411B	OWC	LT	Man	WRAIR	Texas	
503	VIS	LC/D	Dog #5	WRAIR	Oklahoma	WR373R
603	NWC	LBB	Man	WRAIR	Brazil	LTB564A
606	NWC	LBB	Man	WRAIR	Brazil	LTB560
609N	NWC	LBB	Man	WRAIR	Brazil	LTB559
618	NWC	LBB & BP	Man	WRAIR	Honduras	H-12
618N	NWC	LBB & BP	Man	WRAIR	Honduras	H-12
621	NWC	LBB & BP	Man	WRAIR	Honduras	See WR620
621N	NWC	LBB & BP	Man	WRAIR	Honduras	See WR620
626N	NWC	LBP	Man	WRAIR	Panama	
626BN	NWC	LBP	Man	WRAIR	Panama	
628	NWC	LBP	Man	WRAIR	Panama	
655	NWV	LC	Man	WRAIR	-	
656	NWC	LBP	Man	WRAIR	-	
657*	OWV	LD	Man	WRAIR	India	MHOM/W/80/DD8
658*	OWV	LI	Man	WRAIR	-	LEM235
661*	OWC	LMJ	Man	WRAIR	-	SASKH
662*	OWC	LMJ	Man	WRAIR	Israel	MHOM/IC/67/JerII
664*	OWC	LT	Man	WRAIR	?	K27
666*	OWC	LAE	Man	WRAIR	Ethiopia	MHOM/ET/72/L100
667*	NWC	LMM	-	WRAIR	Belize	BEL 21
668*	NWC	LMM	Nyctomys	WRAIR	Belize	MNYC/BZ/74/M379
669*	NWC	LMA	-	WRAIR	-	M2269
670*	NWC	LMA	Sandfly	WRAIR	Brazil	IFLA/BR/67/Ph8
671*	NWC	LPIF	Man	WRAIR	Venezuela	MHOM/VE/57/LL1
673*	NWC	LGAR	-	WRAIR	-	JAP78
675*	NWC	LBB	Man	WRAIR	Brazil	MHOM/BR/75/M2903
676*	NWC	LBP	Man	WRAIR	Panama	MHOM/PA/71/L594
677*	NWC	LBG	Man	WRAIR	Brazil	MHOM/BR/75/M4147
678*		LEN	-	WRAIR	-	L88
679*		LHH	Coendou	WRAIR	Panama	MCOE/PA/65/C8
681*		LHD	-	WRAIR	-	GML3
682*		LGE	Gerbili	WRAIR	-	GERBILLI
683*	OWC	LT	Man	WRAIR	USSR	MHOM/SO/60/LRC-L39
684*	OWV	LD	Man	WRAIR	Ethiopia	MHOM/ET/67/L83
685*	NWC	LC	Man	WRAIR	Brazil	MHOM/BR/74/M2682
3948B	NWC	LBB?	-	WRAIR	-	-
674	NWC	LMM	CAT	WRAIR	-	
675N	NWC	LBB	Man	WRAIR	Brazil	MHOM/BR/75/M2903
710	VIS	LC/D	-	WRAIR	-	
663*	OWC	LMJ	Man	WRAIR	-	
R125		?	-	WRAIR	-	
R170	OWC	LT?	-	WRAIR	-	
R300A		LT & LMA	-	WRAIR	-	
R300B		LD & LMA	-	WRAIR	-	
R335	OWC	LT?	-	WRAIR	-	
TX HAMS	NWC	LMA-G	-	WRAIR	-	
672	NWC	LMA-G	Man	WRAIR	-	

TABLE 2. CAE isolate identification - continued.

WRAIR No.	Category	Parasite Species	Host Species	Source	Locality	Other Designation
CL017B	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL017B
CL019A	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/84/CL019A
CL020B	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/84/CL020B
CL025	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/84/CL025
CL029	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL029
CL046	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL046
CL047	NWC	LBG	Man	Corredor	Colombia	MHOM/CO/85/CL047
CL048	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL048
CL049	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL049
CL052	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL052
CL058	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL058
CL060	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL060
CL063	NWC	LBB	Man	Corredor	Colombia	MHOM/CO/85/CL063
CL064	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL064
CL066	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL066
CL069	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL069
CL070	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL070
CL072	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL072
CL073	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL073
CL079	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL079
CL080	NWC	LBB	Man	Corredor	Colombia	MHOM/CO/85/CL080
CL082	NWC	LBG	Man	Corredor	Colombia	MHOM/CO/85/CL082
CL083	NWC	LBB	Man	Corredor	Colombia	MHOM/CO/85/CL083
CL085	NWC	LBP	Man	Corredor	Colombia	MHOM/CO/85/CL085
K038	OWC	LMJ	-	Keithly	-	
K039	NWC	LBG	-	Keithly	-	
K041	NWC	LBB	-	Keithly	-	
K042	NWC	LBP	-	Keithly	-	
K043	NWC	LBP	-	Keithly	-	

[1]Walter Reed isolates

[2]Colombia isolates

[3]Keithly isolates

[4]NIH isolates

[5]P. Jackson isolates

[6]Preliminary CAE identification

*WHO controls

LMM-P - Leishmania mexicana mexicana - pifanoi
LMA-G - L. m. amazonensis - garnhami
LGAR - L. garnhami
LPIF - L. pifanoi
LBB - L. braziliensis braziliensis
LBP - L. b. panamensis
LBG - L. b. guyanensis
LT - L. tropica
LMJ - L. major
LAE - L. aethiopica
LD - L. donovani
LC - L. chagasi
LI - L. infantum
LEN - L. enrietti
LHH - L. hertigi hertigi
LHD - L. h. deani
LGE - L. gerbilli

TABLE 3. Geographical diversity of Leishmania subspecies associated with intensity of study.

Locale	Species Diversity	Number of Isolates Characterized*
Panama	BP, BB, MA/MG, C, AR, HH, MM/MP	83
Colombia	BP, MM/MP, MA/MG, C, BB, BG	50
Brazil	BP, BB, BG, MA/MG, C	37
Belize	BP, BB, MA/MG, MM/MP	14
Kenya	D, MJ	14
Ethiopia	D, AE	9
India	D	6
USA	MM/MP, MA/MG	5
Costa Rica	MM/MP, BG, MA/MG	4
Venezuela	MM/MP, MA/MG	4
Dominican Republic	MM/MP	2
Israel	MJ	2
Peru	BB, MM/MP	2
Afghanistan	T	1
Iraq	T	1
Surinam	BG	1
USSR	T	1

TABLE 4. Numbers of isolates in each subspecies or species of Leishmania tested in this study. Note: 20 of each type should be required to produce a valid profile of the group. Isolates are from various laboratories.

Species or Subspecies*	Number of Isolates
LBP	104
LBB	56
LMM	43
LMA	47
LC	27
LD Old World	28
LMJ Old World	32
LT Old World	25
LBG	28
LME	2
LAE Old World	7
LAR	1
LMP	2
LMG	2
LHH	4
LHD	1
LGE	1

*At present it is not possible to separate LMM from LMP and LMA from LMG.

TABLE 5. Conditions for electrophoresis and components for developing zymograms.

Enzyme	Cell Buffer	Membrane** Buffer	Voltage	Run Time (Minutes)	Reaction Buffer	Substrate/Stain Components*
GOT	1 or 2	1:14	180	15	A or D	Substrate: 100 mg L-Aspartic acid; 75 mg α-Keto-glutaric acid, readjust to pH 8.0; add 10 mg Pyridoxal-5-phosphate; pour this mixture with agar*** by itself in petri dishes. Stain: (second petri dish) 75 mg Fast blue BB.
GPI	1 or 2	1:14	180	15	B	20 mg Fructose-6-phosphate; 15 mg MTT Tetrazolium; 15 mg β-TPN (Na salt); 10 mg Phenazine methosulfate (PMS); 60 mg EDTA; 25 units (1 flake) Glucose-6-phosphate dehydrogenase (Bakers yeast); 120 mg $MgCl_2$.
GSR$_1$[a]	3	1:9	180	12	C	30 mg Oxidized Glutathione; 5 mg β-NADPH; < 1 mg 2, 6-Dichlorophenol-indophenol; 15 mg MTT.
GSR$_2$[a]	3	1:9	200	15	C	30 mg Oxidized Glutathione; 5 mg β-NADH; < 1 mg 2, 6-Dichlorophenol-indophenol; 15 mg MTT.
ICD[a]	3	1:14	180	15	D	100 mg DL-Isocitric acid (Na_3), readjust to pH 8.0; add 15 mg MTT; 15 mg β-NADP; 10 mg PMS.
MDH[b]	4	1:9	200	15	E	15 mg oxalacetic acetic; 15 mg β-NADH (Na_2 salt).
ME	1 or 2	1:14	180	15	F	270 mg DL-Malic acid; 604 mg Tris; 15 mg MTT; 15 mg β-TPN (Na salt); 10 mg PMS; 25 mg $MnCl_2$.
MPI	5	1:5	160	15	G	15 mg Mannose-6-phosphate; 10 mg β-NADP (Na_2); 10 mg MTT; 5 mg PMS; 1 mg Glucose phosphate isomerase; 15 units (1 flake) Glucose-6-phosphate dehydrogenase; 40 mg $MgCl_2$.
6-PGDH[a]	3	1:14	180	15	B	15 mg 6-Phosphogluconic acid (Na_3 salt); 15 mg MTT; 15 mg β-TPN; 10 mg PMS; 60 mg EDTA (Na_2 salt); 120 mg $MgCl_2$.

Cell buffers:

1. 0.1 M. Tris (12.11 g/L)/0.1 M Maleic acid (11.62 g/L)/0.01 M EDTA (Na_2)(2.92 g/L)/0.01 M $MgCl_2$ (2.03 g/L); adjust to pH 7.4 with 40% NaOH.
2. 0.1 M Tris (12.11 g/L)/0.05 M Maleic Acid (5.81 g/L)/0.01 M EDTA (2.92 g/L)/0.11 M Sodium phosphate dibasic (16 g/L), pH 7.4.
3. 0.2 M Phosphate buffer: 7.1 g Na_2HPO_4/3.24 g NaH_2PO_4 in 385 mL distilled water, pH 7.0.
4. 0.29 M Tris (28.12 g/L)/0.09 M Citric acid monohydrate (1.89 g/L), pH 7.0.
5. 0.05 M Tris (6.06 g/L)/0.05 M NaH_2PO_4 (6.0 g/L), pH 7.5.

Reaction buffers:

A. 0.1 M Tris (12.11 g/L), adjust to pH 8.0 with 50% HCl.
B. 0.06 M Tris (7.28 g/L)/0.04 M Sodium phosphate monobasic (4.72 g/L), pH 8.0.
C. 0.25 M Tris (30.24 g/L)/0.103 M Sodium phosphate monobasic (12.34 g/L), pH 8.4.
D. 0.1 M Tris (12.11 g/L)/0.069 M Sodium phosphate monobasic (8.21 g/L), pH 8.0.
E. 0.018 M Sodium phosphate monobasic/0.082 M Sodium phosphate dibasic, pH 7.4.
F. 0.06 M Tris (7.28 g/L)/0.057 M Sodium phosphate monobasic 6.88 g/L), pH 7.5.
G. 0.1 M Tris (12.11 g/L)/0.101 M Sodium phosphate monobasic (12.12 g/L), pH 7.5.

Number of applications of the aliquot to the cellulose acetate plate:

Dilutions are made using 1 part cell buffer either buffer 1 or 2 and 14 parts distilled water.
1:1 dilution then 1X: GPI, ME, MPI.
1X from lysate: GOT, GSR_1, GSR_2, MDH, 6-PGDH.
2X from lysate: ICD

*To make 50 mL of stain (about 6 petri dishes). All chemicals from Sigma.

**The membrane buffers are dilutions of 1 part cell buffer: distilled water.

***The CA plates after electrophoresis are placed on substrate petri dishes for + 12 min. at 37°C; then blotted dry and placed on the stain petri dishes in which the bands are monitored.

[a]These systems require cooling during electrophoresis.

[b]This system requires viewing with Ultra-Violet light for the bands to be visible.

TABLE 6. Biochemical similarities among certain Leishmania subspecies and species. Som similarities are based on data from one isolate and at present must remain suspect. See Figure 2 for a graphic extension of these data.

BP	BG	BB		MJ	T	AE	
-	89	59	BP	-	31	33	MJ
	-	58	BG		-	33	T
		-	BB			-	AE

MA-G	AR	MM-P	ME	
-	53	32	11	MA-G
	-	18	7	AR
		-	11	MM-P
			-	ME

Figure 1. Diagrammatic representations of the electrophoretic patterns of GPI for various Leishmania subspecies.

Note: Two bands for a subspecies indicates two different allomorphs or natural polymorphism.

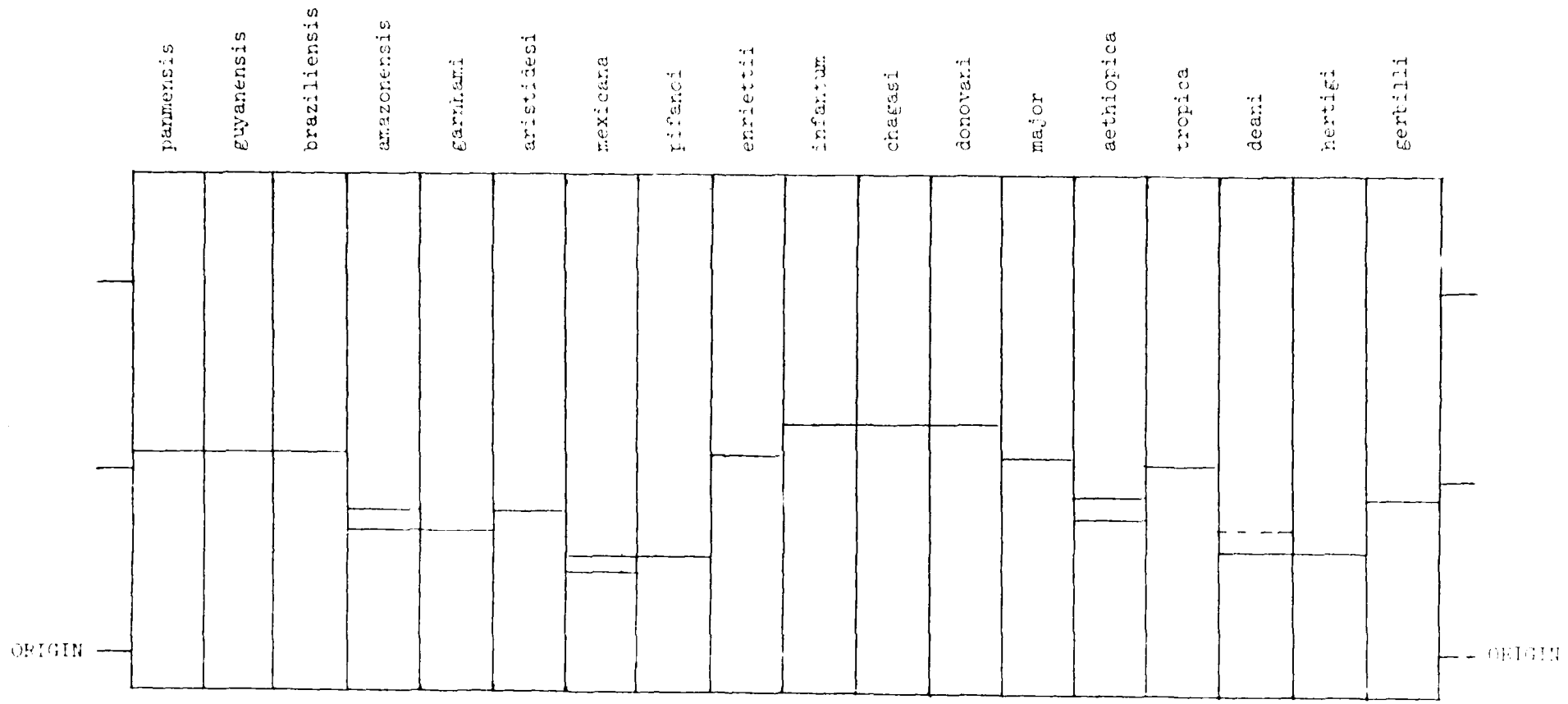
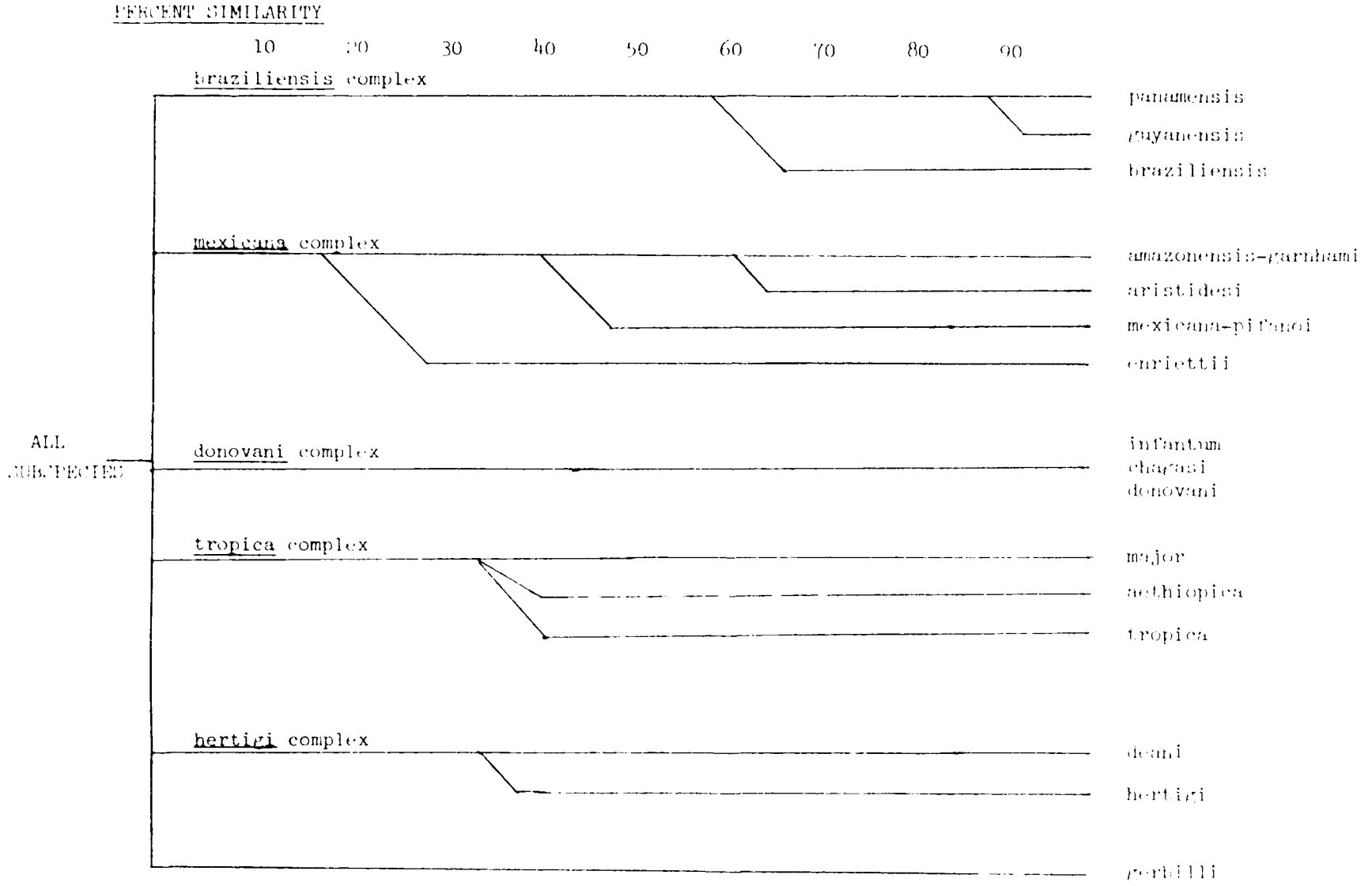


Figure 2. Cladogram grouping of subspecies of Leishmania based on their levels of enzyme profile similarities as noted in Table 6.



LITERATURE CITED

1. Avise, J. C. 1974. Systematic value of electrophoretic data. Syst. Zoo. 23:465-481.

2. Ayala, F. J. 1975. Genetic differentiation during the speciation process. Evol. Biol.., 8:1-78.

3. Ayala, F. J., M. L. Tracey, D. Hedgecock, and R. C. Richmond. 1974. Genetic differentiation during the speciatio process in Drosophila. Evolution 28:576-592.

4. Myers, J. H. 1978. Isozymes and allozymes: alternate forms of protein adaptation? Can. J. Genet. Cytol. 20:187-192.

5. Chance, M. L. 1979. The identification of Leishmania. Symp. Brit. Soc. Parasit. 17:55-74.

6. Chance, M. L., L. F. Schnur, S. C. Thomas and W. Peters. 1978. The biochemical and serological taxonomy of Leishmania from the Aethiopian zoogeographical region of Africa. Ann. Trop. Med. Parasitol., 72:533-542.

7. Kreutzer, R. D. and H. A. Christensen. 1980. Characterization of Leishmania spp. by isozyme electrophoresis. Amer. J. Trop. Med. Hyg., 29:199-208.

8. Miles, M. A., M. M. Pova, A. A. Souza, R. Lainson and J. J. Shaw. 1979. Some methods for the enzymatic characterization of Latin-American Leishmania with particular reference to Leishmania mexicana amazonensis and subspecies of Leishmania hertigi. Roy. Soc. Trop. Med. Hyg., 74:243-252.

9. Miles, M. A., R. Lainson, J. J. Shaw, M. M. Povoa and A. A. Souza. 1981. Leishmaniasis in Brazil: XV. Biochemical distinction of Leishmania mexicana amazonensis, L. braziliensis braziliensis and L. b. guayanensis - aetiological agents of cutaneous leishmaniasis in the Amazon Basin of Brazil. Trans. Roy. Soc. Trop. Med. Hyg., 75:524-529.

10. Aljeboori, T. J. and D. A. Evans. 1980. Leishmania spp. in Iraq. Electrophoretic isoenzyme patterns. I. Visceral leishmaniasis. Trans. Roy. Soc. Trop. Med. Hyg., 74:169-177.

11. Kreutzer, R. D., M. E. Semko, L. D. Hendricks and N. Wright. 1983. Genetic characterization and identification of Leishmania spp. by isozyme electrophoresis. Am. J. Trop. Med. Hyg., 703-715.

12. Lainson, R. 1983. Leishmaniasis. In: Handbook Series in Zoonoses. Ed. J. H. Steele, pp. 41-103.

13. 1982. Biochemical Characterization of Leishmania. Chance and Walton Eds. pp. 280.

14. Kreutzer, R. D., N. Souraty, P. B. McGreevy and E. D. Franke. In review. A New World Leishmania which can cause either cutaneous or diffuse cutaneous leishmaniasis in human hosts. Am. J. Trop. Med. Hyg.

15. Bryceson, A. D. 1972. Immunological aspects of cutaneous leishmaniasis, Essays on Tropical Dermatology, Vol. 2, Ed. J. Marshall, Amsterdam, Excerpta Medica, 230-241.

16. Hommel, M. 1978. The genus Leishmania: Biology of the parasites and clinical aspects. Bull. L. institut Pasteur, Vol. 75, 5-102.

17. Marsden, P. D. 1979. Current concepts in parasitology: Leishmaniasis. New Engl. J. Med.:350-352.

18. Lainson, R. 1982. Leishmaniasis, Handb. Ser. in Zoon. Sec. C, Vol. 1, 41-103.

19. Lainson, R. 1983. The American leishmaniasis: some observations on their ecology and epidemiology. Roy. Soc. Trop. Med. Hyg., 77:569-596.

20. Miles, M. A. 1983. Biochemical identification of the leishmanias. Proc. 3rd Venez. Cong. Micro. Symp. Leish. In Press.

21. Bonfante-Garrido, R. 1983. Observaciones sobre Leish. mex. ven. Proc. 3rd Venez. Cong. Micro. Symp. Leish. In Press.

22. Kreutzer, R. D. and N. Souraty. Accurate identification of Leishmania isolates by study of three enzymes. Annual meeting Am. Soc. Trop. Med. Hyg.

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